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13. ABSTRACT *(Maximum 200 words)*

ErbB2/neu amplification and overexpression is the most common genetic alteration in breast cancer. As a growth factor receptor and member of the EGFR family, ErbB2 signaling is dependent upon target tissue expression of ErbB2 regulating growth factors and other EGFR family members. To determine the role ErbB2 plays in normal and malignant mammary gland development I examined expression of ErbB2 agonists by ISH during normal development of the mouse mammary gland. This analysis has been completed for NRG1 and βC. NRG1 is expressed within the stroma of both virgin and pregnant mammary glands. βC on the other hand is expressed within stroma of virgin glands and epithelial cells during pregnancy. This result suggests that βC has differing functions during mammary gland development. To determine the role signaling by ErbB2 and ErbB4 plays in mammary gland development I expressed dominant negative forms of these receptors as a transgene within the developing mammary gland. These results indicate that ErbB2 is required for normal lobuloalveolar development, while both ErbB2 and ErbB4 play important roles in lactation at parturition. Thus these two receptors may be coupled at parturition.

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INTRODUCTION

Breast cancer is the most commonly diagnosed cancer and the second most frequent cause of cancer mortality in North American women (1). Normal and malignant mammary epithelial growth is regulated by the activities of circulating hormones including estrogen, progesterone, and prolactin (2, 3) and locally produced growth factors (4, 5). Perturbation of signaling cascades regulated by these mammary growth modulators can lead to malignant transformation of the breast.

One important mediator of growth factor signaling, the receptor tyrosine kinase ErbB2/HER2/Neu (referred to here as ErbB2) is amplified and overexpressed in 15-40% of human breast carcinomas (6). Overexpression of ErbB2 is associated with poor prognosis, especially in patients with lymph node involvement (6, 7, 8). In phase III trials of a recombinant humanized anti-ErbB2 monoclonal antibody, targeting of breast cancer cells overexpressing ErbB2 results in tumor growth inhibition and regression (reviewed in: (9)). These results suggest that ErbB2 is a suitable target for therapeutic treatment of breast cancer patients with tumors overexpressing ErbB2.

The proposed role of ErbB2 signaling in breast cancer was further substantiated in transgenic mouse models of breast cancer. Overexpression of mutationally activated ErbB2 in the mammary epithelium of transgenic mice induces multifocal breast carcinomas (10, 11). Similarly, overexpression of wild-type ErbB2 results in mammary tumor development and metastasis after a long latency (12). Breast tumors isolated from these latter mice have novel activating deletions within the ErbB2 ectodomain (13, 14), suggesting that mutational activation of overexpressed ErbB2 may be an important event in breast cancer.

ErbB2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases (15). Additional members of this family are EGFR itself, ErbB3 (16) and ErbB4 (17). There are at least nine different genes that encode growth factors belonging to the EGF family. The growth factors can be placed into three functional groups based upon the ability to bind to and activate distinct sets of receptors. For example, EGF and transforming growth factor α (TGFα) bind to and activate EGFR, whereas, neuregulin (NRG)1 and NRG2 bind to and activate ErbB3 and ErbB4 (18, 19, 20). The third functional group includes betacellulin (BC), epiregulin (ER), and heparin-binding EGF-like factor (Hb-EGF); these

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growth factors bind to EGFR and ErbB4 (21, 22, 23) (also reviewed in: (24)).

A ligand which binds to and activates ErbB2 has not been identified. Significantly, ErbB2 signaling can be activated through heterodimer formation with an activated EGFR family member. For example, EGF does not bind to ErbB2 but can activate ErbB2 only when ErbB2 is co-expressed with EGFR (25, 26). Although, ligand activated EGFR family members can form an active signaling dimer with any other EGFR family member, ErbB2 appears to be the preferred heterodimer partner (27, 28). In fact ligand stimulated receptors compete for dimer formation with ErbB2 (29, 30). Moreover, heterodimer formation with ErbB2 increases ligand affinity (26, 31) and prolongs receptor signaling (30, 32). The unique signaling activites of each receptor:ligand combination means that diverse cellular responses can be mediated through heterodimer formation with ErbB2 (20, 22, 23). Taken together, these results support a signaling paradigm in which ErbB2 fulfills a role as central mediator of EGFR family signal transduction.

Expression of ErbB2, its receptor signaling partners, and several ErbB2 agonists has been detected within the developing rodent mammary gland. ErbB2 is expressed within stromal and epithelial cells of virgin mouse mammary glands and lobuloalveolar epithelium from pregnant and lactating mice (4, 33). Similar cellular and temporal expression patterns have been documented for EGFR (4, 33, 34), ErbB3 (35), and ErbB4 (35). Likewise, the ErbB2 agonists EGF (33, 36), TGFα (33, 36), and AR (33, 37) are expressed in both virgin and pregnant mammary tissue, whereas, significant levels of NRG1 expression is only detected during pregnancy (35). The omnipresence of EGFR family members and ligands reflects the multiple functions of this receptor family during mammary gland development.

Distinct biological responses of mammary tissue to signaling by the EGFR family are regulated by both the target receptors and activating ligands. For example, substantial evidence supports a role for EGFR and its activating ligands in ductal proliferation and morphogenesis during virgin mammary development. Mammary implants containing EGF (36, 38), TGFα (36), or AR (39) induce ductal morphogenesis and TEB formation in ovariectomized mice. In addition, transgenic expression of AR (39) or TGFα (40, 41) results in extensive mammary ductal hyperplasia and, in TGFα expressing mice, progression to mammary carcinoma. Consistent with the proliferative effects of EGFR ligands on mammary development,

inhibition of EGFR signaling, through expression of a dominant negative EGFR mutant, results in decreased ductal proliferation and morphogenesis in the virgin mammary gland (42).

In contrast to EGFR and its ligands, signaling induced by NRG1, a ligand for ErbB3 and ErbB4, is associated with mammary differentiation. In the AU565 human breast cancer cell line NRG1 induces a differentiation phenotype (43, 44) which requires ErbB2 expression (45). *In vivo*, mammary implants containing NRG1, but not TGFα, induce ductal differentiation into milk-secreting lobuloalveoli (46). Taken together, these results suggest that NRG1 induces distinct biological responses from TGFα and expression of NRG1 during pregnancy may be coupled to ErbB2 signaling during mammary epithelial differentiation.

The exact function of ErbB2 signaling during normal mammary gland development has not been established. Coupling of ErbB2 signaling with individual EGFR family members means that ErbB2 could play an important functional role in both the proliferative phase of virgin mammary development and mammary differentiation during pregnancy and lactation. Knowledge of the mechanisms which govern ErbB2 mediated breast development may provide valuable insights into the regulation of ErbB2 signaling in breast cancer. We therefore employed a mutant ErbB2 receptor, with dominant negative activity, to inactivate endogenous ErbB2 signaling during mouse mammary gland development. The results demonstrate that ErbB2 signaling plays an important role in the terminal stages of lobuloalveolar development and lactational at parturition.

A similar dominant negative receptor mutant was used to determine the role of ErbB4 during mammary gland development. Preliminary results indicate that, like ErbB2, ErbB4 signaling is required for epithelial differentiation and lactation at parturition. In contrast to dominant negative ErbB2, lobuloalveolar expansion appears to occur normally in dominant negative ErbB4 expressing mice. This result suggests that ErbB2 and ErbB4 have similar but non-overlapping signaling functions in the mammary gland. Based upon work presented in this report, a model for ErbB2 and ErbB4 signaling during mammary gland development and in breast cancer is proposed.

BODY

Materials and Methods

Plasmids

The plasmid pMMTV-ErbB2ΔIC containing the, mouse mammary tumor virus (MMTV) LTR driven, rat ErbB2 cDNA with C-terminal truncation and hemagglutinin (HA) epitope-tag (Figure 1) was constructed in two steps as follows. First, an intermediate plasmid was generated by trimolecular ligation of *Eco*RI digested pcDL-SRα296 (47), the 5' ca. 2.4 kb *Eco*RI-*Nde*I fragment from the rat ErbB2 cDNA bearing plasmid pSV2neuN (48), and the 236 bp *Nde*I-*Eco*RI digested PCR product generated from pSV2neuN using a forward oligonucleotide primer upstream of the unique *Nde*I site and the reverse primer 5'-GAATGAATTCAGGCGTAATCAGGCACATCGTATGGGTACAGCCTACGCATCGTATA C-3'. The resultant plasmid, pN-DID-HA, contains a truncated rat ErbB2 cDNA, encoding residues 1 through 694, followed by an in-frame HA (12CA5) epitope-tag (49, 50) and C-terminal *Eco*RI site. The modified rat ErbB2 cDNA was then subcloned, to generate the plasmid pMMTV-ErbB2ΔIC, via a trimolecular ligation involving *Hind*III-*Eco*RI digested pMMTV-Sv40-Bssk (pMMTV-GAL4/236-SV40 minus the *GAL4*/236 gene)(51) generously provided by Philip Leder through Baulin Li, the 530 bp *Hind*III-*Aat*II digested PCR product generated from pN-DID-HA using the forward primer with a 5' *Hind*III linker 5'-CTAAGCTTCAATGATCATCATGGAGCT-3' and the reverse primer 5'-GGGGCACAAGGTGGACAGGC-3', and the 3' ca.1.6 kb *Aat*II-*Eco*RI fragment from pN-DID-HA.

A similar strategy was used to construct the MMTV driven dominant negative ErbB4 receptor (ErbB4ΔIC) with two tandem C-terminal Flag epitope tags. A trimolecular ligation was performed with HindIII (site filled with T4 DNA pol) EcoRI digested pMMTV-Sv40-Bssk, SalI (site filled with T4 DNA pol) SpeI digested pLXSN-ErbB4 (20), and the SpeI EcoR1 digested PCR product from the following primer pair: 5' CCCACTAGTCATGAC and 5' CGGAATTCTTATTACTTGTCACTGTCGTCCTTGTAGTCCTTGTCATCGTCGTCCTTG TAGTCGGGTGCTGTGCC.

The plasmid pBI-ErbB2ΔIC, which served as a riboprobe template specific for truncated and HA-tagged rat ErbB2, was generated as follows. pMMTV-ErbB2ΔIC was digested with *Nde*I, the restriction site was filled in with T4 DNA polymerase, and the linearized plasmid was digested with *Eco*RI. The 236 bp 3' *Nde*I-*Eco*RI fragment was ligated to *Sma*I-*Eco*RI

digested pBluescript I S/K (Stratagene) destroying the *Nde*I and *Sma*I sites in the process.

Generation of transgenic mice

For microinjection, pMMTV-ErbB2ΔIC and pMMTV-ErbB4ΔIC were digested at unique *Sal*I-*Spe*I sites and the ca. 6.3 kb fragment was separated from vector sequences by agarose gel electrophoresis followed by purification with a gel extraction kit (Qiagen). The purified DNA fragment was microinjected into single-cell B6SJL/F2 zygotes at a concentration of 12 μg/ml in 10 mM Tris, pH 7.5, 0.1 mM EDTA by Carole Pelletier under the direction of Dr. David Brownstein at the Transgenic Mouse Shared Resource of the Yale University School of Medicine.

Identification of transgenic mice by PCR

Transgenic mice were identified by PCR analysis of DNA isolated from tail biopsies. Briefly, tail segments biopsied from 3 week old weaned mice were incubated overnight at 55°C in 50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% SDS, and 500 μg/ml proteinase K. The protealized tails were phenol/chloroform extracted, nucleic acids were precipitated with EtOH, and the final pellet was resuspended into 10 mM Tris, pH 7.5, 1 mM EDTA. PCR analysis using 32 cycles at 94°C for 1 min 15 s, 56°C for 2 min 15 s, and 72°C for 3 min 15 s in a PTC-100 programmable thermal cycler (M. J. Research, Inc.) was performed with ca. 2 μg of tail DNA in a final volume of 100 μl containing 10 mM Tris, pH 9.0, 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each doeoxynucleotide, 0.15 μM of each primer, and 2.5 U *Taq* polymerase (Boehringer Mannheim). Primers for the amplification of a 763 bp MMTV-ErbB2ΔIC fragment were 5'-GGCCTGGGGATCCACTCGCTGGGG-3' (corresponding to nucleotides 1364 to 1388 of the rat *ErbB2* ORF) and 5'-GGCGTAATCAGGCACATCGTATGG-3' which hybridizes to the 3' terminal HA 12CA5 epitope of MMTV-ErbB2ΔIC. Primers for the amplification of a ca. 300bp MMTV-ErbB4ΔIC fragment were 5'-CGGAATTCTTACTTGTCATCGTCG and 5'-CAAGTATGCTGATCCAGATCGGGA. Primers for the amplification of a ca. 500 bp mouse *β-casein* exon 7 fragment served as a PCR internal control and have been described elsewhere (52). PCR products were analyzed on a 1% agarose gel.

Riboprobe synthesis and purification

Where possible buffers used for riboprobe synthesis and transcript purification were pretreated with DEPC using standard procedures. For RNase protection and in situ hybridization experiments DNA template was linearized for anti-sense riboprobe synthesis by restriction digest to

completion with an excess of enzyme. Contaminating ribonucleases were inactivated by protease treatment of the linearized DNA. Briefly, the digested DNA was phenol/chloroform extracted, EtOH precipitated, and washed extensively with 70% EtOH, prior to and following protease treatment. Protease treatment was performed at 37°C for 1 hr in 10 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.6% SDS, and 150 µg/ml protease K. The final purified DNA pellet was resuspended in ddH_2O at a concentration of 1 µg/ml. In vitro transcription and subsequent DNase treatment were performed using a MAXIscript in vitro transcription kit (Ambion) with 1 µg of template DNA exactly as described by the manufacturer. For in situ hybridization 5 µl of ^{35}S UTP was used in the transcription reaction in place of ^{32}P dUTP.

For riboprobe purification, an equal volume of gel loading buffer II (Ambion) was added to the DNase-treated transcription reaction and transcripts were fractionated on a 6% polyacrylamide, 8 M Urea gel prepared with Sequel NE reagents as described by the manufacturer (American Bioanalytical). Full-length transcript was excised and eluted from the gel slice into 700 µl of 500 mM NH_4OAc, 1 mM EDTA, and 0.1% SDS at 37°C for 3 hrs.

RNA isolation and RNase protection assay

For isolation of mammary gland RNA, ca. 0.2 g of tissue from the number 4 and/or number 5 inguinal mammary gland was snap frozen in liquid N_2 and stored at -70°C until use. RNA was isolated from frozen tissue by homogenization in 4.0 ml of TRIzol (Gibco-BRL) with a glass homogenizer using a type B pestle (Janke and Kunkel KIKA-Labortechnik) and the exact procedure as described by the manufacturer. Isolated RNA was resuspended into DEPC (Sigma) treated ddH_2O and stored at -70°C until use.

For RNase protection analysis, 20 µg of total RNA was precipitated with 5 X 10^5 cpm of specific riboprobe and dried under vacuum. The same amount of t-RNA (Gibco-BRL) precipitated with riboprobe served as a negative control. Riboprobe hybridization, RNA digestion, and isolation of RNA:probe hybrids, were performed using the RPA II ribonuclease protection assay kit (Ambion) exactly as described by the manufacturer. Probe protected RNA fragments were separated on a 6% polyacrylamide 8 M urea gel and, without additional processing, the gel was exposed to XAR-5 (Kodak) x-ray film without intensifying screens at -70°C for 1-7 days.

Whole-mount staining of mouse mammary glands
The entire number 4 inguinal mammary gland was spread onto a glass microscope slide, fixed in acidic ethanol and stained in carmine solution exactly as described previously (46).

Tissue preparation for histological analysis
For hematoxylin/eosin staining, in situ hybridization, and immunohistochemistry a portion of the number four inguinal mammary gland was spread onto a glass microscope slide and fixed in freshly prepared 4% paraformaldehyde in PBS overnight at 4°C. The fixed tissue was rinsed in ddH_2O and partially dehydrated through a graded EtOH series to 70%. The samples were embedded in paraffin and 6 μm sections were dried onto gelatin-coated slides using standard procedures at the Yale University Department of Pathology Critical Technology Unit.

Immunohistochemistry
For immunohistochemical staining, paraffin embedded mammary gland tissue was dewaxed in xylene, hydrated through a descending ethanol series, and washed in PBS. Endogenous peroxidase activity was inactivated by incubating the sections in 0.5% H_2O_2 in PBS for 15 min at room temperature followed by two washes in PBS for 5 min per wash. Non-specific binding sites were blocked for 30 min. at room temperature with 10% rabbit serum or 10% goat serum in PBS if the secondary biotinylated antibody was produced in rabbit or goat, respectively.

For immunodetection of HA-tagged dominant negative ErbB2, sections were incubated with 50 μl of 10% rabbit serum in PBS containing 1 μg of anti-HA high affinity rat monoclonal antibody (Boehringer Mannheim) overnight at 4°C, then 50 μl of 10% rabbit serum in PBS containing 1 μg of biotinylated rabbit anti-rat (Vector), and finally 50 μl biotin-avidin-peroxidase complex generated in PBS using a Vectastain Elite ABC kit (Vector), as described by the manufacturer, for 1 hr at room temperature. Between each reagent, the sections were washed 3 times in PBS for 15 min per wash. Peroxidase activity was detected by incubating the sections for 7 min. with 200 μl of DAB reagent (Vector), prepared according to the manufacturer's instructions, followed by a brief wash in PBS. Sections were lightly counterstained in hematoxylin (Polysciences) for 10 sec. according to the manufacturers instructions, dehydrated through an ascending ethanol series, cleared in xylene, and coversliped with permount (Fisher).

In situ hybridization

In situ hybridization was performed using [^{35}S]-UTP labeled riboprobes on 6 μm paraffin or frozen sections of mammary glands from developmentally staged female mice. Sections were deparaffinized and dehydrated as described for immunohistochemistry. The sections were defatted by incubating with chloroform for 5 min and hydrated through a descending ethanol series. The hydrated sections were washed in PBS for 5 min and protease treated for 10 min at 37°C in PBS containing 2 μg/ml protease K. The treated slides were rinsed in PBS, post-fixed in 4% paraformaldehyde in PBS for 10 min, quenched with 0.2% glycine in PBS for 5 min, and washed in PBS for 5 min. Non-specific binding sites were blocked by incubating the sections for 10 min in 100 mM triethanolamine (Sigma), pH 8.0, 0.9% NaCl, containing 0.25% acetic anhydride (Sigma). The slides were washed in 2X SSC (20X SSC = 3 M NaCl, 0.3 M Na-citrate, pH 7.0) for 5 min, dehydrated through an ascending ethanol series, treated with chloroform for 5 min, washed two times with 100% ethanol for 2 min per wash, and air-dried. Hybridization mixture contained 10 mM Tris, pH 7.5, 600 mM NaCl, 2 mM EDTA, 10 mM DTT, 1X Denhardt's (Sigma), 500 μg/ml total yeast RNA (Ambion), 100 μg/ml poly-A (Pharmacia), 100 mg/ml dextrane sulfate (Sigma), 50% deionized formamide (Ambion), and 4 X10^4 cpm/μl of [^{35}S]-UTP labeled riboprobe and was heated at 80°C for 10 min immediately before use. To each section 50 μl of hybridization mixture was applied, the sections were overlaid with parafilm coverslips, and hybridized at 50°C for 16 hrs in a humid chamber containing 10 mM Tris, pH 7.5, 600 mM NaCl, 2 mM EDTA, and 50% formamide (Sigma). Following hybridization, the parafilm coverslips were removed with tweezers and the slides were washed twice at low-stringency for 15 min per wash at 50°C in 2X SSC, 50% formamide, 0.1% β-mercaptoethanol. Unhybridized probe was digested by placing the slides in 10 mM Tris, pH 8.0, 500 mM NaCl, containing 20 μg/ml of RNase A (Sigma) for 30 min at 37°C. The low-stringency washes were repeated and the slides were washed an additional two times in 0.1X SSC, 1% β-mercaptoethanol at 50°C for 15 min per wash. The slides were dehydrated through an ascending ethanol series, with a final concentration of 600 mM NaCl included in ethanol solutions under 80%, and air-dried. Completely dried slides were dipped in Kodak NTB-3 nuclear track emulsion diluted 1:1 with ddH_2O at 45°C and were exposed at 4°C in light-tight slide boxes containing desiccant (Sigma). The slides were warmed to room temperature and developed in Kodak D-19 developer for 2.5 min, washed in ddH_2O for 30 sec, fixed in Kodak Fixer for 3 min, and washed in running tap water for 15 min. The

sections were lightly counterstained with hematoxylin using the same procedure described for immunohistochemistry.

Results

In situ hybridization of staged mouse mammary glands
The temporal and spatial expression of the EGF family of ligands during mammary gland development would provide valuable functional information for these hormones. Therefore, in collaboration with Dr. S. Dey at St Louis University, we have embarked on an extensive analysis of growth factor expression within the mammary gland. Previously, the use of paraffin sections of mammary tissue yielded inconsistent results. This analysis will use frozen sections provided by Dr. Dey. Growth factors for which ISH analysis is in progress are: AR, NRG1, NRG2, NRG3, βC, Hb-EGF and ER. Preliminary data is included in this report.

Expression of NRG1 has been reported within the stroma of mammary glands at late pregnancy (35). The results in Figure 1 are consistent with this data (lower panels, right=dark field, left=light field). Furthermore, we also observe expression of NRG1 within the stroma of mice at 1 day post-partum (top and middle panels [note that middle panel is labeled incorrectly], right=dark field, left=light field) suggesting that NRG may play a role in differentiation at parturition.

βC, a ligand for EGFR and ErbB4, is expressed at high levels within the stroma of 6 wk virgin mice (Figure 2, top panel, right=dark field, left=light field). The expression pattern of βC is dramatically different, however, during pregnancy and at parturition. At these developmental stages βC is expressed at extremely high levels within the lobuloalveolar epithelial cells(middle and bottom panels, right=dark field, left=light field). This may indicate a shift in βC function from the virgin to pregnant mammary gland.

Expression of a truncated ErbB2 receptor
Overexpression of truncated ErbB2, lacking the entire kinase domain and C-terminal tyrosine phosphorylation sites, inhibits normal ErbB2 signaling *in vitro* (53) and can severely compromise ErbB2-mediated tumor formation in the nude mouse (54). We constructed a similar dominant negative mutant by replacing the rat ErbB2 endodomain, downstream of A694, with an influenza virus hemaglutinin (HA) epitope-tag followed by a single translational stop codon. The dominant negative ErbB2 receptor was fused with the MMTV promoter to direct transgene

Neuregulin-1

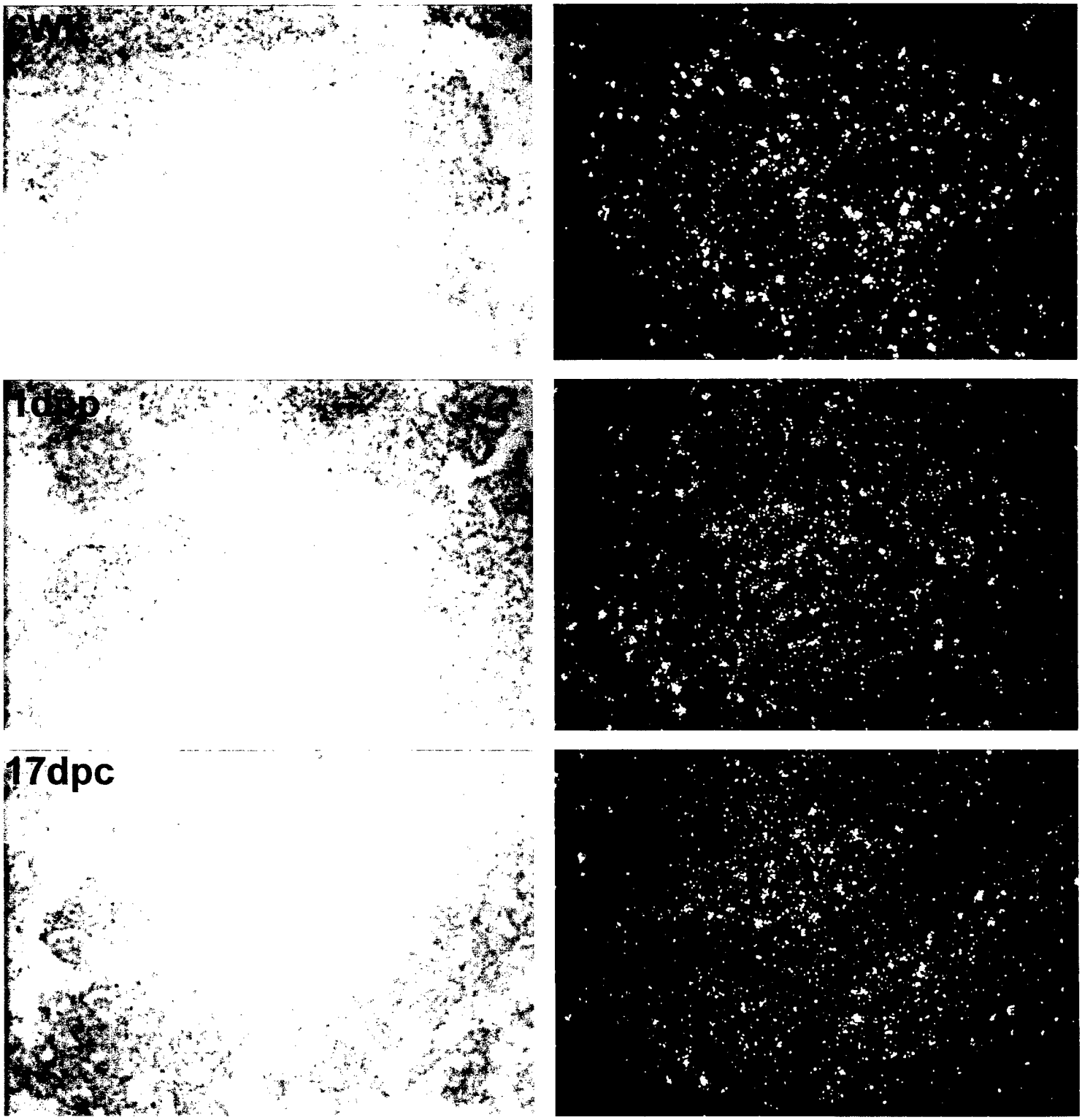

Figure 1. In situ hybridization of staged mouse mammary glands with NRG1.

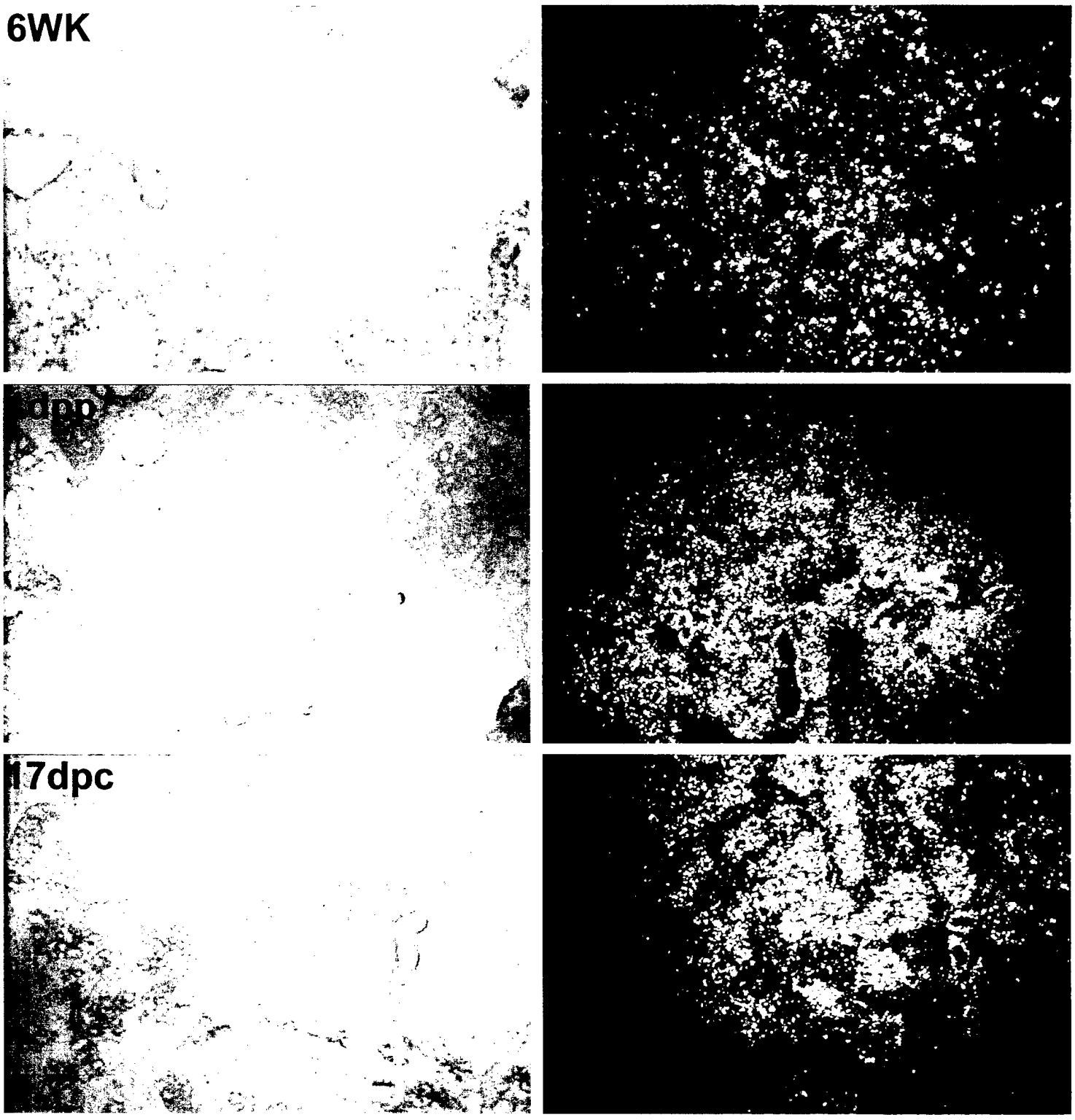

Figure 2. In situ hybridization of staged mouse mammary glands with BC.

expression within mouse mammary epithelial cells. (Figure 3A). The resultant construct, designated MMTV-ErbB2ΔIC, encodes a protein of 704 residues with a predicted molecular mass of 105 KDa. Expression of the correct sized ErbB2ΔIC protein was confirmed in FR3T3 cells transfected with MMTV-ErbB2ΔIC. The 180 KDa endogenous ErbB2 receptor was detected in both the MMTV empty vector and MMTV-ErbB2ΔIC cell lines (Figure 3B lanes 1 and 2). Expression of the ErbB2ΔIC protein, migrating at ca. 110 KDa, was only detected in the MMTV-ErbB2ΔIC cell line (Figure 3B lane 2).

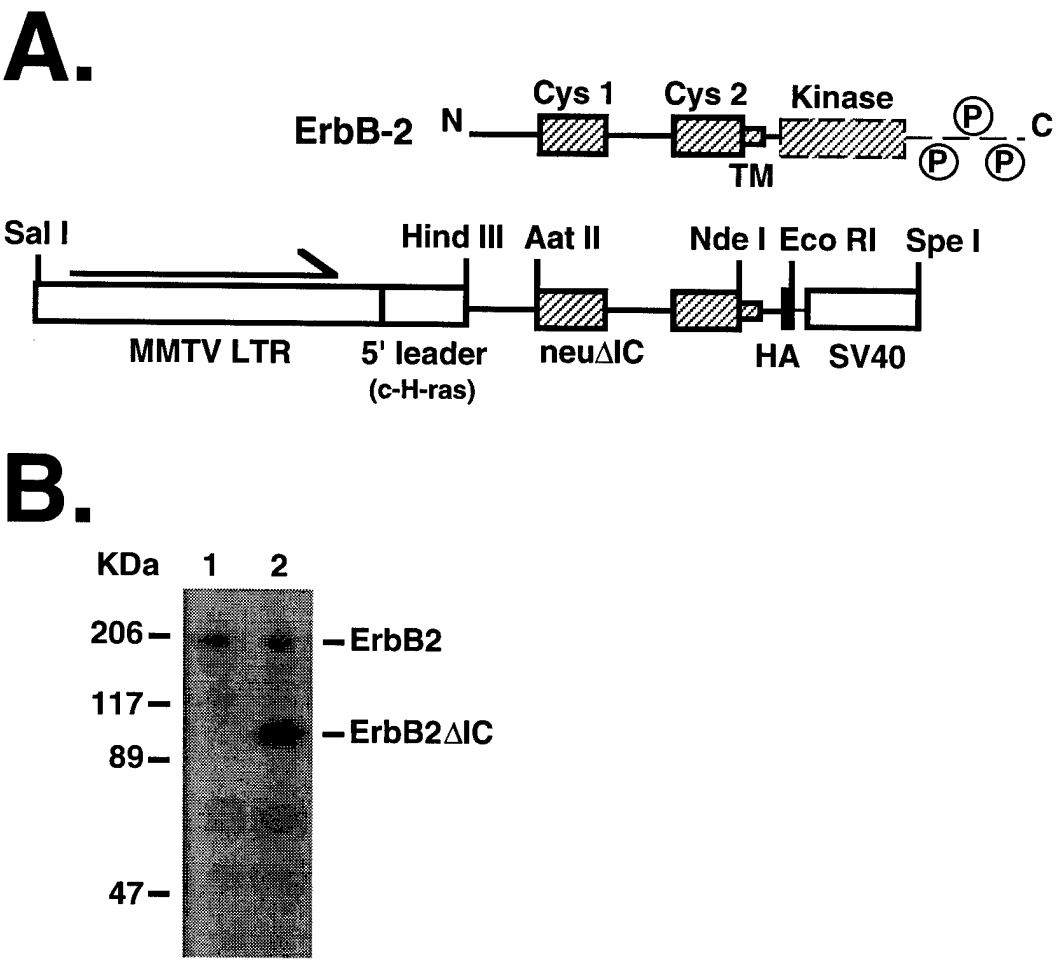

Figure 3. ErbB2ΔIC contruct (A) and expression in transfected cells (B).

Dominant negative activity of MMTV-ErbB4ΔIC

Dominant negative activity for a truncated ErbB4 receptor has not been reported. Therefore, FR-3T3 cells lines stably transfected with MMTV-ErbB4ΔIC were isolated and the ability of ErbB4ΔIC to inhibit EGFR

phosphorylation in βC stimulated cells was determined. Stimulated cells were lysed in the presence of sodium-vanadate, immunoprecipitated for EGFR, and western blotted with antibody 4G10 which recognizes phosphorylated tyrosine residues.

βC stimulated a large increase in EGFR phosphorylation in FR3T3 cells transfected with vector (Figure 4, compare lanes 1 and 3). Although βC stimulated EGFR phosphorylation in cells expressing ErbB4ΔIC the level of phosphorylation was significantly lower than observed in control cells (Figure 4, compare lanes 3 and 4). These results indicate that the MMTV-ErbB4ΔIC construct has dominant negative activity in vitro.

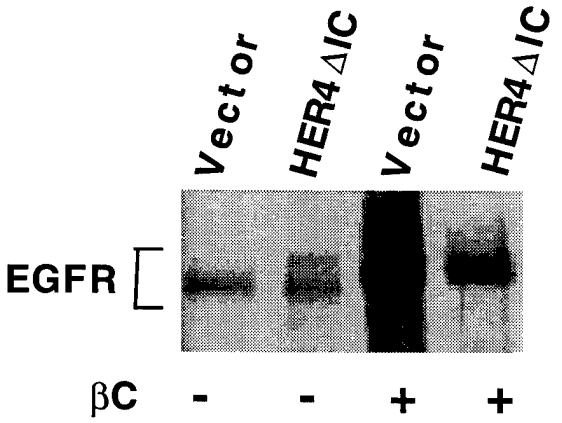

Figure 4. Dominant negative activity of ErbB4ΔIC

Transgenic mice derived from MMTV-ErbB2ΔIC and MMTV-ErbB4ΔIC
To determine the effect of dominant negative ErbB2 and ErbB4 within the developing mammary gland, transgenic mice were derived by injecting MMTV-ErbB2ΔIC or MMTV-ErbB4ΔIC DNA into the pronuclei of fertilized one-cell zygotes from B6SJL/F2 mice. Following implantation of microinjected zygotes into pesudopregnant females, 17 founder mice were delivered. Polymerase chain-reaction (PCR) analysis of tail DNA identified six founders (35%) with MMTV-ErbB2ΔIC transgene integration (data not shown). When crossed into an FVB strain to generate F2 pups, one founder of the above six failed to transmit the transgene to its offspring and the F2 females from another founder line were sterile. Transgene expression by the F2 offspring of the remaining four founders was determined by Northern blot analysis using a ErbB2ΔIC specific probe. F2 females were impregnated by crossing with FVB males and mammary gland RNA was isolated at mid-late pregnancy (15-19 days p.c.). Transgene expression was detected in mammary glands from the two founder transgene lines

designated 5286 and 5289 (data not shown). Transgene expression was not detected in the remaining two founder lines. Phenotypic analysis of mammary glands from mice expressing ErbB2ΔIC was performed on F3 females derived by crossing founder line 5289 F2 mice with FVB strain mice. The 5289 phenotype was confirmed by analysis of founder line 5286.

A similar analysis was performed for ErbB4ΔIC founder mice. Of 53 original founder mice, 18 mice contained the ErbB4ΔIC transgene. When assayed for transgene transmission, 8 mice successfully transmitted the transgene and when assayed by ribonuclease protection assay (RNPA), 6 expressed the transgene in mammary glands from 5 wk old mice (Figure 5). Mice which expressed high levels of the transgene throughout development (founders 5963 and 5997) were used for further phenotypic analysis.

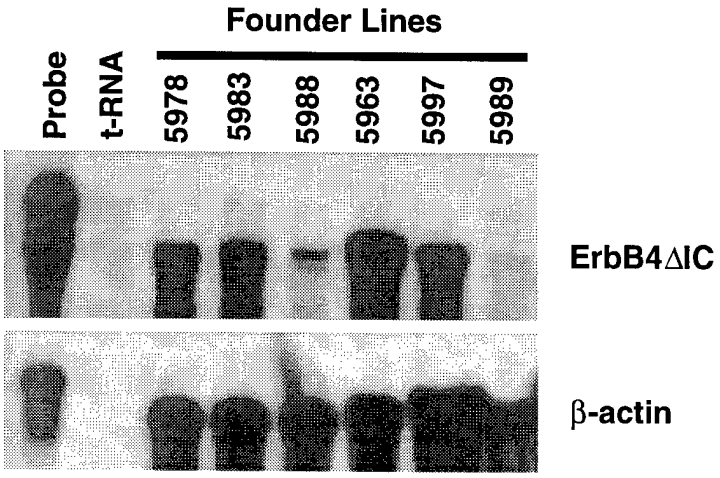

Figure 5. Identification of founder lines expressing ErbB4ΔIC by RNPA.

Mammary gland expression of the ErbB2ΔIC and ErbB4ΔIC transgenes

To determine the temporal expression pattern of ErbB2ΔIC and ErbB4ΔIC, RNA was isolated from the number 4 inguinal mammary gland of developmentally staged female mice and subjected to analysis by RNPA. Significantly, a protected fragment representing endogenous ErbB2 or ErbB4 RNA was not detected in mammary glands from transgenic or control mice: indicating that the levels of endogenous receptor expression were below the sensitivity of this assay. Similar patterns of expression were observed in both the ErbB2ΔIC (Figure 6, top) and ErbB4ΔIC (Figure 6, bottom) transgenic lines. Transgene expression was first detected within virgin females as the mice entered puberty at 5 wks and expression levels increased slightly with age with maximal expression in the mature virgin mammary gland. Expression levels throughout pregnancy were several-fold

greater than observed within the mature virgin mammary gland. The highest level of expression, however, was observed at 1 day post-partum. The window of high level post-partum transgene expression was restricted because a decrease in expression was observed at both 3 and 12 days post-partum. Mammary gland involution resulted in a return of transgene expression to pre-pregnancy levels. Similar protected fragments were never detected in mammary glands from developmentally staged non-transgenic siblings.

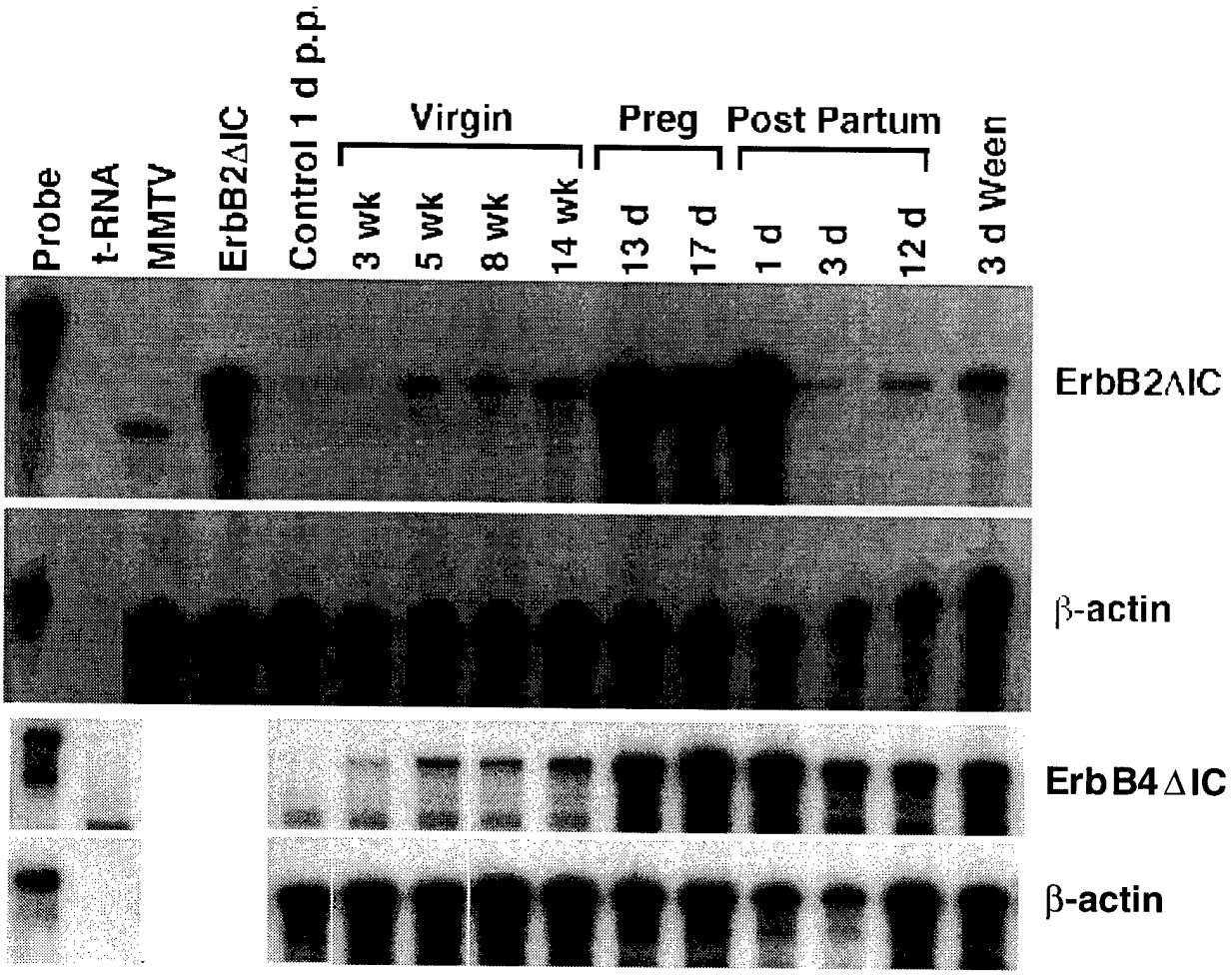

Figure 6. Developmental expression of ErbB2ΔIC and ErbB4ΔIC in mammary glands of transgenic mice.

Mammary gland expression of ErbB2ΔIC or ErbB4ΔIC inhibits lobuloalveolar development at parturition

Mammary gland development can be broadly divided into two hormonally regulated phases: 1) ductal proliferation and branching morphogenesis in the pubescent virgin and 2) lobuloalveolar development and epithelial terminal differentiation during pregnancy and lactation. To determine the

effects of ErbB2ΔIC and ErbB4ΔIC expression on mouse mammary gland development, wholemounts and histologically stained paraffin-embedded sections of mammary glands from ErbB2ΔIC or ErbB4ΔIC expressing virgin mice were examined at 3, 5, 7, 10, and 14 weeks of age. Despite the expression of ErbB2ΔIC and ErbB4ΔIC within the virgin mammary gland after 5 wks (Figure 6), we did not detect effects on terminal end-bud formation, ductal branching, ductal growth, or cellular morphology within the virgin mammary gland (data not shown).

With the onset of pregnancy, proliferation of lateral and terminal ductal epithelial buds initiates ductal side-branching and the accumulation of ductal alveolar units. By 17 days post-coitus proliferation has essentially ceased (55) and lobuloalveoli line the entire ductal system. High levels of ErbB2ΔIC expression during pregnancy had no effect on ductal side-branching or alveolar development up to and including 17 days post-coitus (data not shown). At 19 days post-coitus, some lobuloalveoli are expanded in non-transgenic mice and the ducts become slightly distended (Figure 7A). Evidence for inhibition of alveolar growth in ErbB2ΔIC-expressing mice was first observed at 19 days post-coitus. Although distended ducts are observed in wholemounts of ErbB2ΔIC expressing mice at this stage, the lobuloalveolar of these mice remained condensed (Figure 3B). An analysis of ErbB4ΔIC expressing mice during pregnancy is in progress.

At 1 day post-partum lobuloalveoli of control mice expand and unfold as the alveolar lumens become engorged with proteinacious lactation products (Figure 7C). In control mice the ducts are obscured by the extensive expansion of lobuloalveoli. Likewise, the lobuloalveoli and ducts of multiparous control mice at 1 day post-partum are distended, but to a much greater extent than uniparous control mice (compare Figure 7E to Figure 7C). A dramatic phenotype was observed in ErbB2ΔIC-expressing mice at 1 day post-partum. Alveoli from both uniparous and multiparous transgenic mice remained condensed (Figure 7D and 7F). Furthermore, distended ducts are clearly visible in wholemounts from the ErbB2ΔIC transgenic mice at 1 day post-partum (Figure 7D and 7F), suggesting that lobuloalveolar but not ductal epithelial cells are adversely effected by ErbB2ΔIC expression. The ErbB2ΔIC phenotype observed at 1 day post-partum was accentuated in multiparous females (> 6 pregnancies; compare Figures 7D and 7F), presumably because multiparity results in both higher levels and more uniform transgene expression driven by the MMTV LTR. At 3 days post-partum epithelial proliferation (56) results in further

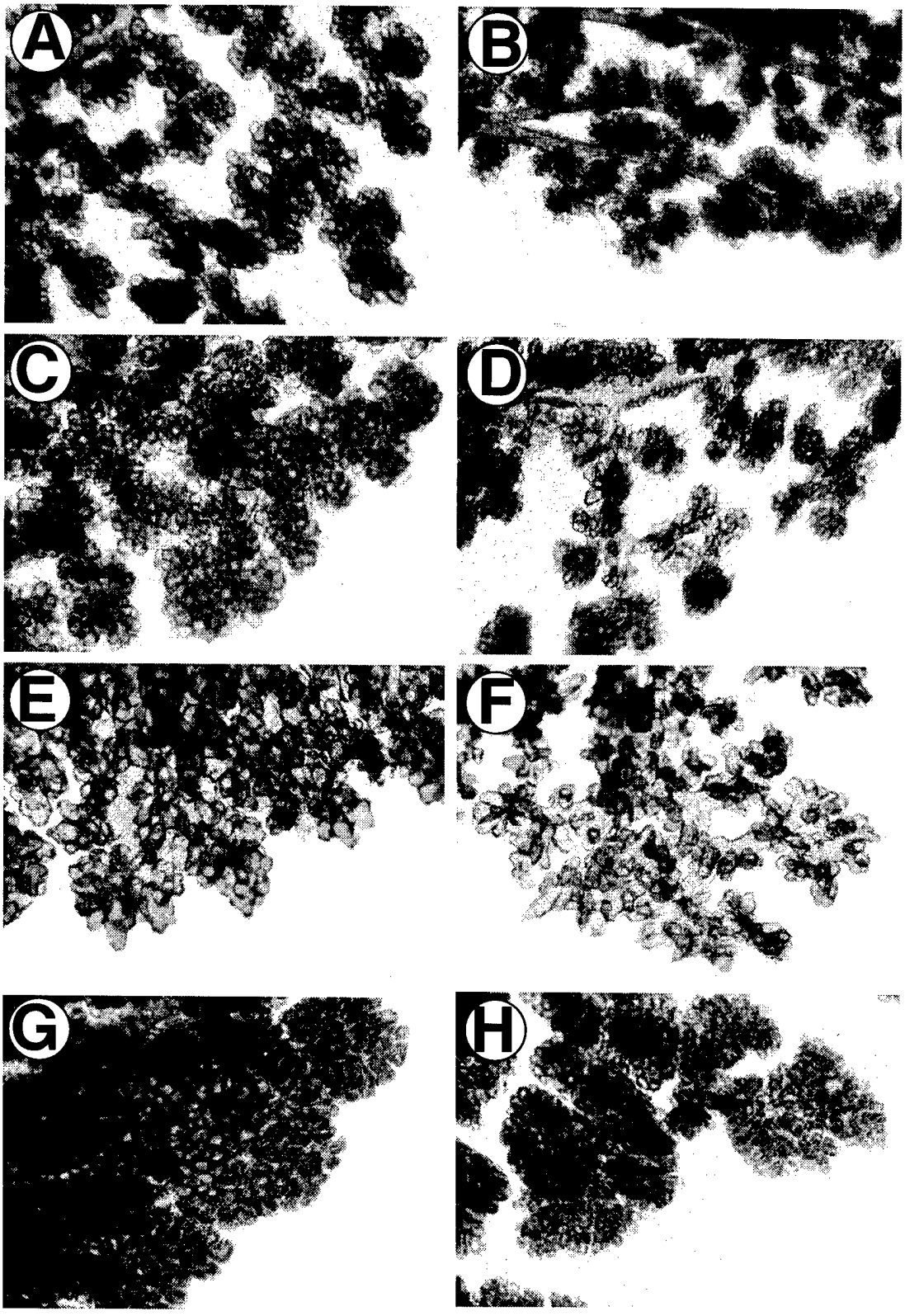

Figure 7. Mammary gland wholemounts from developmentally staged mice expressing ErbB2ΔIC.

accumulation and expansion of lobuloalveoli in control mice (Figure 7G). Although distended lobuloalveoli were prominent within transgenic mice at 3 days post-partum, the lobuloalveoli were still much smaller when compared to control mice (Figure 3H). The recovery of lobuloalveolar development in the ErbB2ΔIC transgenic mice at this stage may be related to the dramatic decrease in transgene expression observed during lactation (Figure 6). Indeed, the ErbB2ΔIC expressing mammary gland was indistinguishable from non-transgenic controls at 12 days post-partum (data not shown): the developmental stage of maximal lactation with a concomitant reduction in transgene expression.

Histological analysis of mammary glands from control mice at 19 days post-coitus revealed expanded lobuloalveoli with the accumulation of cellular and lumenal secretory lipids (arrow, Figure 8A). In some alveoli lumenal proteinacious material representing the early stages of terminal differentiation and lactation was detected (arrowhead, Figure 8A). Although secretory lipids were detected within lobuloalveoli of ErbB2ΔIC expressing mice at 19 days post-coitus (arrow, Figure 8B), the lobuloalveoli were much smaller than control mice. Proteinacious material was not detected in the lumens of lobuloalveoli from ErbB2ΔIC-expressing mice (Figure 8B).

At 1 day post-partum, secretory epithelium lining the lobuloalveoli of control mice are no longer cubiodial, but have a flattened appearance, indicating that the cells have undergone secretory differentiation (Figure 4C). In addition, the lumens of control mice are filled with granular proteinacious material (arrowhead, Figure 8C) and a small amount of lipid (arrows, Figure 8C) indicating that normal milk production is in progress (Figure 8C). In multiparous controls, the lobuloalveoli are much larger than uniparous controls and the lumens were empty due to the efficient secretory process of these glands (Figure 8E). A majority of alveolar epithelium from ErbB2ΔIC expressing mice at 1 day post-partum retained their cuboidal secretory structure observed at 19 days post-coitus (compare Figure 8B and 8D). Lumenal products, present in lobuloalveoli with flattened epithelium, resemble secretory lipids typically observed during late pregnancy (compare arrows of Figure 8D with Figures 8A and 8B). Multiparity dramatically exaggerated the inhibitory effect of ErbB2ΔIC expression on lobuloalveolar development. Condensed lobuloalveoli with cuboidal epithelium predominated in mammary glands from multiparous transgenic females at 1 day post-partum (Figure 8F). At 3 days post-partum the alveolar epithelium of both transgenic and control mice are rounded as they enter the first phase of post-partum

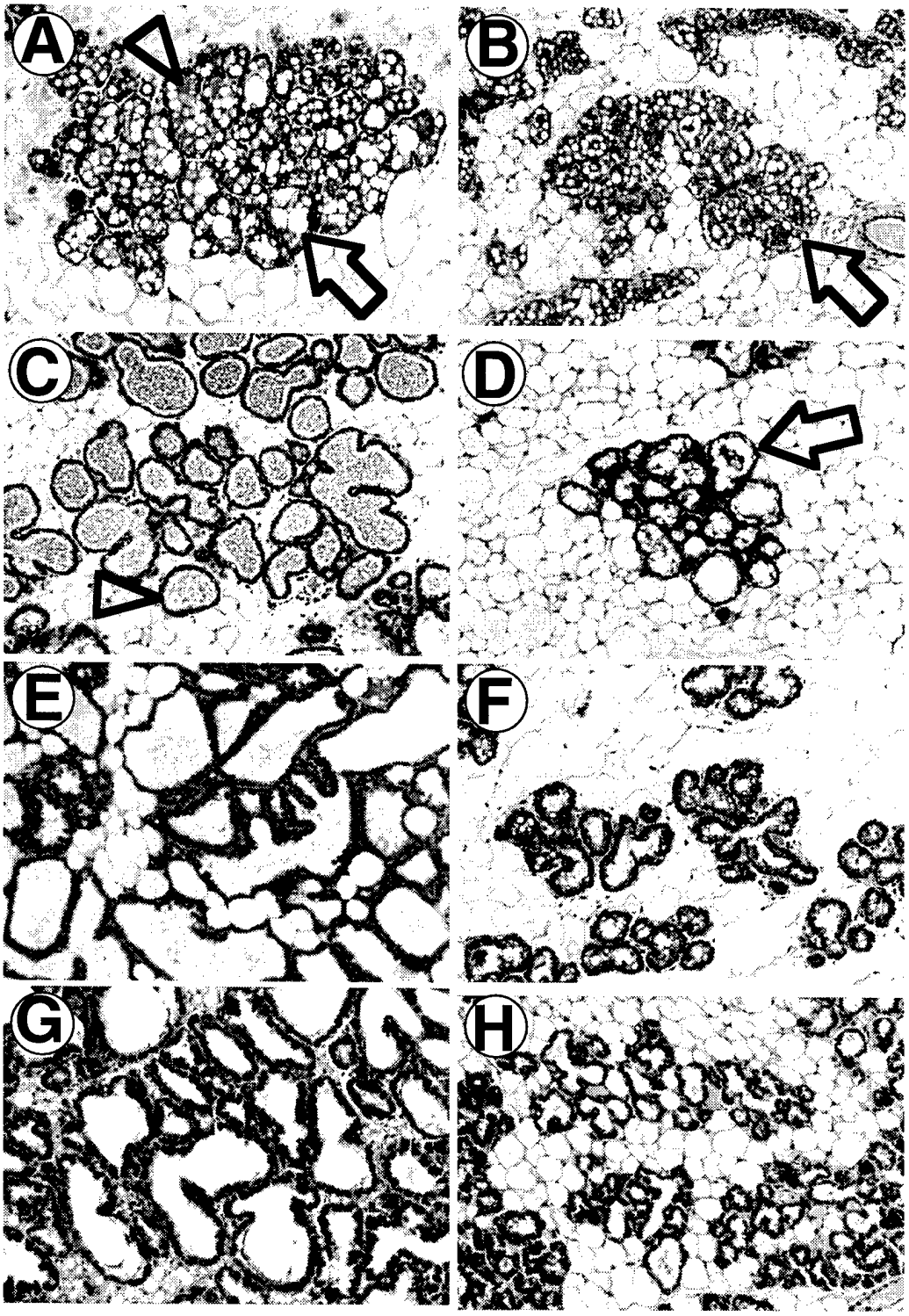

Figure 8. H/E stained paraffin sections of developmentally staged mammary glands from ErbB2ΔIC.

proliferation (56)(Figures 8G and 8H). Despite the apparent recovery of epithelial cell function, lobuloalveoli of ErbB2ΔIC expressing mice at 3 days post-partum were still much smaller than non-transgenic controls.

Expression of ErbB4ΔIC within mammary glands at 1 day post-partum had no effect on lobuloalveolar expansion and unfolding (Figure 9A). When examined histologically, however, the majority of lobuloalveoli from ErbB4ΔIC expressing mice lacked lactation products The lactation products that were detected consisted of lipid rather than the proteinacious material typical of this developmental stage (Figure 9B, compare to control Figure 8C).

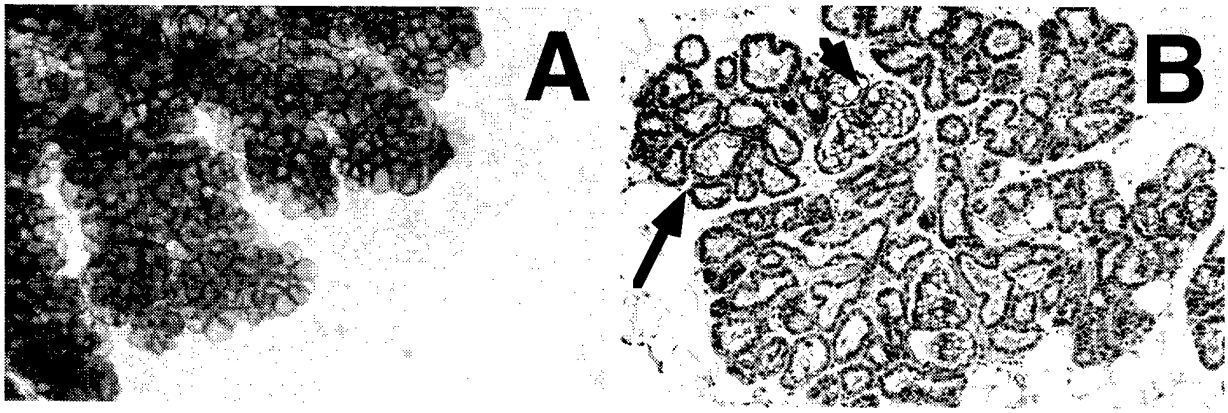

Figure 9. Wholemount (A) and paraffin section (B) of mammary gland from ErbB4ΔIC at 1 day post-partum.

Taken together these results provide evidence that signaling by both ErbB2 and ErbB4 play important non-overlapping roles in lobuloalveolar development and lactation at parturition.

ErbB2ΔIC expression is associated with non-secreting lobuloalveoli

In contrast to the uniform structure of lactating mammary glands from non-transgenic siblings, mammary glands from ErbB2ΔIC-expressing mice displayed a high degree of intragland heterogeneity (compare Figure 8A and 8B). Alveolar clusters with cuboidal epithelial cells always lacked lumenal secretory products. In contrast, other alveolar clusters with slightly flattened epithelium contained secretory lipids typically observed during pregnancy. To determine if this heterogeneous structure was related to ErbB2ΔIC expression levels transgene expression was determined by immunohistochemistry (IHC).

Anti-HA, which recognizes the HA epitope-tagged ErbB2ΔIC, yielded specific membrane staining of epithelium from condensed lobuloalveolar clusters (arrows Figure 10B). When compared to the same region of an adjacent section stained with hematoxylin-eosin, the lumens of ErbB2ΔIC staining lobuloalveolar clusters lacked secretory products (compare arrows of Figure 10A and 10B). IHC staining of ErbB2ΔIC was never observed in epithelium of lobuloalveolar clusters which contained secretory products (arrowheads, Figure 10A and 10B) or non-transgenic controls (data not shown). These results demonstrate a direct concordance between detectable levels of ErbB2ΔIC expression and the inhibition of lobuloalveolar epithelial differentiation and secretory activity at 1 day post-partum.

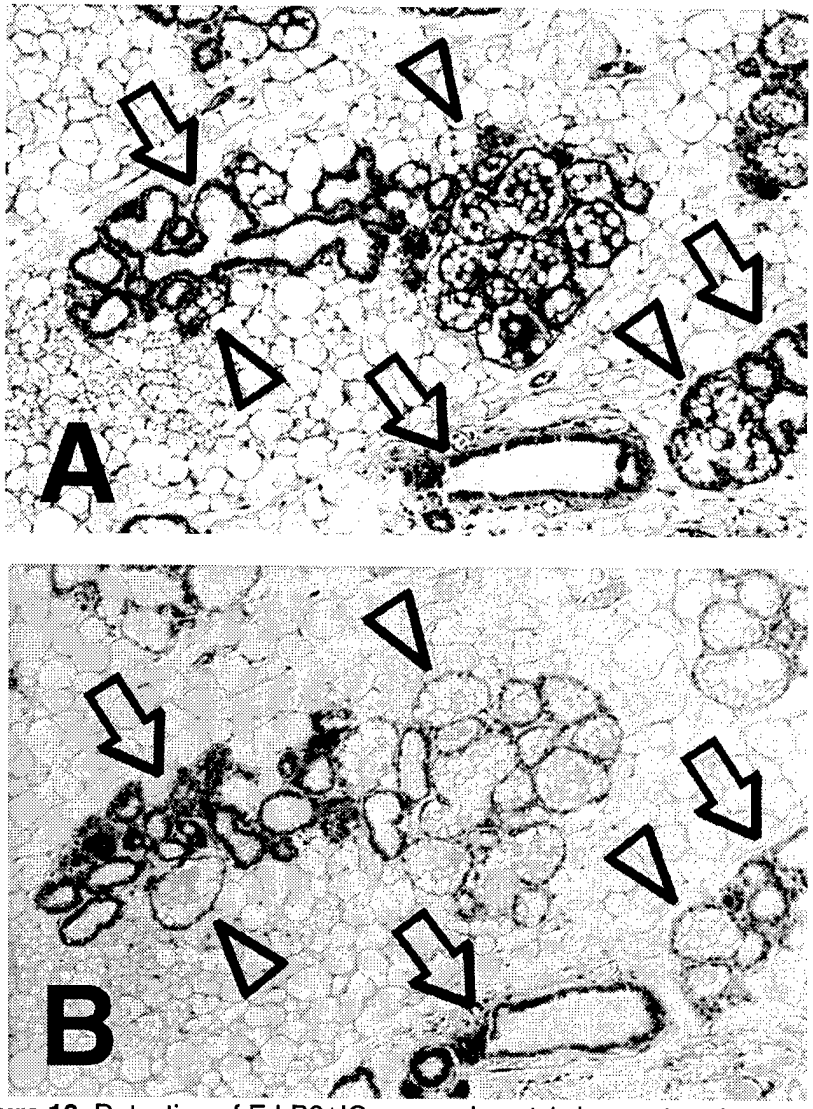

Figure 10. Detection of ErbB2ΔIC expression at 1 day post-partum. (A) H/E stained section. (B) Anti-HA immunohistochemistry.

Despite the dramatic inhibition of lobuloalveolar development and secretory processes observed within mammary glands of ErbB2ΔIC and ErbB4ΔIC expressing mice at parturition, milk was clearly visible within the stomachs of each pup throughout lactation and there was no apparent consequences of ErbB2ΔIC or ErbB4ΔIC expression on nursing pups (data not shown). Incomplete inhibition of terminal differentiation, resulting in functionally lactating ErbB2ΔIC-expressing mammary glands, may be a result of the variegated transgene expression observed at 1 day post-partum (Figure 5B).

Discussion

Locally synthesized EGF family hormones and their receptors play important roles in normal and neoplastic mammary development (4, 5). Indeed, a large body of evidence indicates that the EGFR family member ErbB2 is an important determinant in the ontogenesis and progression of human breast cancer (reviewed in: (6)). In an attempt to inactivate normal ErbB2 signaling during mammary development, I directed expression of a dominant negative ErbB2 receptor (ErbB2ΔIC) to the developing mouse mammary gland. Although levels of ErbB2ΔIC expression was several fold greater than endogenous ErbB2 throughout mouse mammary gland development, the first ErbB2ΔIC specific phenotype was observed at late gestation (19 days post-coitus). A minor defect in lobuloalveolar expansion was detected at this developmental stage. At parturition, a majority of the condensed alveoli of ErbB2ΔIC expressing mice failed to undergo normal expansion and unfolding into distended, lactationally active, lobuloalveoli. Furthermore, IHC analysis revealed a direct concordance between high levels of ErbB2ΔIC protein expression and a lack of lobuloalveolar secretory products. Preliminary results indicate that ErbB4 also plays a role in lactation at parturition. In contrast to ErbB2, however, ErbB4ΔIC expressing mice did not affect lobuloalveolar expansion. These results suggest that ErbB2 plays an important role in the terminal stages of lobuloalveolar development and both ErbB2 and ErbB4 are required for normal lactational activity at parturition. To my knowledge, this is the first report describing a function for signaling by an EGF family receptor in mammary epithelial differentiation during pregnancy or lactation.

A similar MMTV driven dominant negative strategy has been used to inactivate EGFR signaling in the developing mouse mammary gland (42). Dominant negative EGFR expression inhibited mammary ductal

morphogenesis in the pubescent virgin mouse. A mammary gland phenotype was not observed in these mice during pregnancy or lactation, apparently due to the relatively high levels of endogenous EGFR. The authors concluded that EGFR plays a role in the proliferative stages of mammary gland development. Although ErbB2 is expressed within the developing virgin mammary gland, my results suggest that ErbB2 signaling does not contribute to epithelial proliferation at this developmental stage.

An ErbB2ΔIC phenotype was also absent during early or mid-pregnancy: the second, and most pronounced, stage of mammary epithelial proliferation (55). The lack of a mammary phenotype in both virgin and pregnant mice suggests that ErbB2 signaling is dispensable during periods of mammary epithelial proliferation. Consistent with our results, EGF injected directly into pregnant mice stimulated levels of mammary gland ErbB2 tyrosine phosphorylation several-fold lower than EGFR (33). These results suggest that the important signaling receptor during pregnancy is EGFR and that ErbB2 signaling plays at best a minor role during this developmental stage.

ErbB2 signaling may be coupled to NRG1 activated ErbB3 or ErbB4 or both at parturition. Expression of NRG1α, within mammary mesenchyme increases dramatically during late pregnancy (35). Consistent with this result, I have previously reported that NRG1 implanted within mammary glands of virgin female mice, induced the formation of alveoli with an accumulation of the milk protein β-casein (46). Significantly, injection of NRG1β into mice at 2 days post-partum selectively induced a large increase of ErbB2 and ErbB3 phosphorylation; suggesting that ErbB2 and ErbB3 are functionally coupled at this developmental stage (33). Preliminary results, demonstrating that ErbB4ΔIC abrogates lactation at parturition, suggests that ErbB4 signaling may be coupled to ErbB2 at this developmental stage. Taken together, these results support a model of receptor signaling in the mouse mammary gland at parturition, where NRG1α produced in the mammary mesenchyme acts in a juxtacrine fashion to stimulate coupling of ErbB2, ErbB3, and possibly ErbB4, signaling pathways within the epithelium of adjacent lobuloalveoli. Presumably, these signal transduction pathways would provide the signals necessary to induce mammary epithelial cell differentiation at the onset of lactation.

The integrated progression of mammary gland development has been investigated at the molecular level using gene disruption strategies. The phenotype at parturition observed in ErbB2ΔIC and ErbB4ΔIC expressing

mice most closely resembles defects in Stat5 and LAR knockout mice. Stat5 is a transcription factor which is phosphorylated and activated by a wide variety of cytokines and hormones (reviewed in:(57, 58)). Phosphorylation of both Stat5 isoforms (a and b) increases dramatically within mammary tissue during late pregnancy and is maintained through the early stages of lactation (59). Consistent with these observations, Stat5a is required for normal lobuloalveolar development and lactation (60, 61). Significantly, Stat5 can be activated by signaling pathways of the EGFR family (62, 63). Likewise, ErbB2 or ErbB4, or both may activate Stat5 at parturition.

The LAR receptor tyrosine phosphatase may regulate ErbB2 signaling at parturition through dephosphorylation of an ErbB2 negative regulatory tyrosine. Mammary glands of mice lacking LAR undergo normal development through pregnancy, however, at 1 day post-partum the lobuloalveoli remain condensed and in some mice fail to secrete lactation products (64). The negative regulatory tyrosine phosphate (residue Y1028) of ErbB2 (65) may be a target of LAR activity. According to this model, removal of the ErbB2 regulatory tyrosine phosphate, at late pregnancy or early post-partum, would allow full activation of ErbB2 signaling pathways during the early stages of lactation. Interestingly, LAR expression is regulated during mammary development (64) and parallels ErbB2 activation observed at late pregnancy and early post-partum (33). The possible coupling of ErbB2 signaling with the signaling pathways of Stat5 and LAR or both, deserves further investigation.

Substantial evidence exists which supports the mitogenic activity of ErbB2 in the development and progression of human breast cancer (reviewed in: (6)). Results presented in this report, however, demonstrate that ErbB2 signaling within the mouse mammary gland may be coupled to ErbB4 to induce epithelial differentiation and lactation at parturition. These functionally distinct responses of mammary tissue to ErbB2 activation can be explained through differential activation and coupling of ErbB2 signaling with other EGFR family members. For example, receptors linked to mitogenic signaling pathways within mammary tissue, including amplified and overexpressed ErbB2 alone or in combination with EGFR, would result in an aggressive mammary carcinoma associated with poor prognosis. Whereas coupling of ErbB2 signaling with a receptor associated with favorable prognostic indicators, such as ErbB4 (66), may indicate a less aggressive mammary tumor. Consistent with this hypothesis, the results described herein indicate that ErbB4 signaling plays an important role during mammary epithelial differentiation and is coupled to ErbB2 signaling at parturition. Therefore, one would predict that coexpression of

ErbB2 and ErbB4 would be an indicator of a less aggressive differentiating mammary tumor.

Characterization of signaling components coupled with ErbB2 activation during mammary epithelial differentiation may lead to the identification of favorable prognostic indicators associated with ErbB2 mediated mammary carcinogenesis. Furthermore, ErbB2 signaling pathways coupled to mammary differentiation could be invoked to antagonize ErbB2 activation within an aggressively proliferating tumor.

CONCLUSIONS

The long-term goal of the research described in this report is to determine the role ErbB2 signaling plays in breast cancer development. To address this issue I have proposed to characterize the expression profiles and the functions of ErbB2 agonists and receptor signaling partners within the mouse mammary gland. To this end I have initiated a large scale ISH experiment to analyze the cellular expression of several ErbB2 agonists within the developing mammary gland. In addition, dominant negative receptors have been expressed as transgenes to identify the functional roles of ErbB2 and ErbB4 signaling in the mouse mammary gland. Both receptors play an important role in lobuloalveolar function and lactation at parturition. I have previously demonstrated that the ErbB4 ligand, NRG1, implanted within mammary glands of virgin female mice, induced the formation of alveoli with an accumulation of the milk protein β-casein (46). Therefore, NRG1 may induce ErbB4:ErbB2 heterodimer formation at parturition and the combined signaling of these receptors may be required to induce epithelial differentiation and lactation at parturition.

Based upon these results the following model of ErbB2 signaling within the mammary gland is proposed (Figure 11). During normal mammary gland development, ErbB2 is coupled to ErbB4 by NRG1 and plays a role in mammary epithelial differentiation and lactation at parturition. This may involve regulation of signaling by LAR or Stat5 or both. Aberrant expression of ErbB2 in undifferentiated mammary epithelial cells, however, results in the coupling of ErbB2 signaling to the mitogenic pathways of EGFR and its ligands in the virgin mammary gland. In this way ErbB2 signaling, probably through Grb-2 or MAPK or both, would contribute to the development and progression of breast cancer. One strong prediction follows that signaling molecules coupled directly to or downstream of activated ErbB2 during mammary differentiation may be ectopically expressed in breast cancer cells to antagonize ErbB2 mitogenic signaling. Identification of molecules coupled to ErbB2 signaling during mammary

differentiation will be one focus of my research during the next year. Based upon the results reported here, the most logical candidate for an ErbB2 antagonist would be ErbB4.

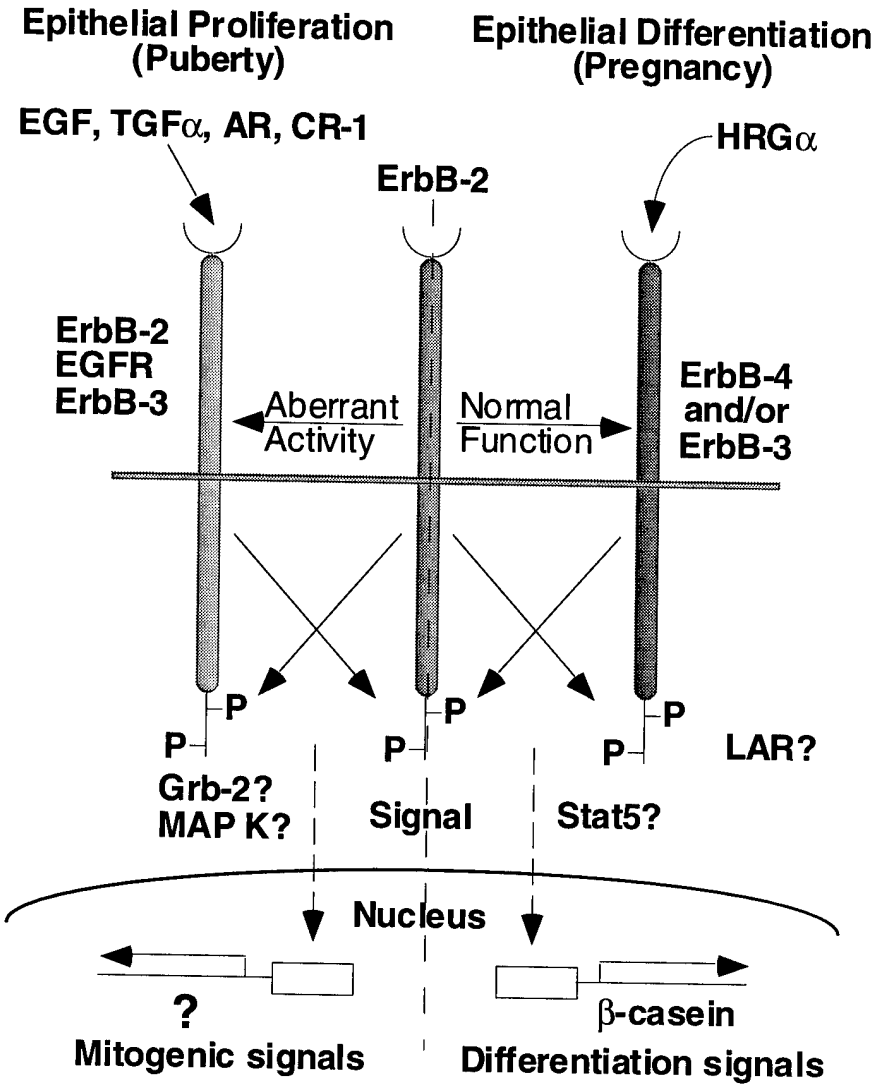

Figure 11. Model of ErbB2 signaling in normal and neoplastic breast development.

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APPENDIX

Personnel Receiving Pay

Frank Jones

Bibliography of Meeting Abstracts

Basic and Clinical Aspects of Breast Cancer
Keystone, Colorado
March 7-12, 1997
Abstract title: Expression of epidermal growth factor receptor family members and their ligands in the developing mouse mammary gland.

Modes of EGF Receptor Signaling
Ames, Iowa
September 25-28, 1997

Abstract title: Dominant negative ErbB-2/neu receptor impairs mammary gland development.

Concordance with Statement of Work

The following is a descriptive summary of the work completed to date and its relation to the proposed Statement of Work. Explanations for aims or tasks not completed are provided where appropriate.

Technical Objective 1. Developmental expression of receptors and growth factors.

Task 1. Quantitative RT-PCR of growth factors from normal and ErbB2 overexposing staged mouse mammary tissue.
Status: This work has been completed by another laboratory and published (33).

Task 2. Perform quantitative RT-PCR analysis in hormonally manipulated mice.
Status: This work will be pursued by Schroeder and Lee (33).

Task 3. *In situ* hybridization analysis of receptors and growth factors identified in mouse tissue during Task 1.
Status: I am performing these experiments now and reported preliminary results within this report.

Technical Objective 2. Response of mammary epithelium to growth factor implants.

Task 4. NRGα and NRGβ implants - complete work in progress.
Status: Work completed.

Task 5. Analysis of implants containing growth factors identified in Task 1 in normal mice.
Status: Isolation of bioactive NRG2, NRG3, and βC will be performed for implant analysis.

Task 6. Analysis of growth factors in hormonally manipulated mice.
Status: Not yet initiated.

Task 7. Analysis of growth factors in ErbB2 overexposing mice during tumor-free latency period.
Status: Due to expense of growth factors and mosaic expression of the ErbB2 transgene in mouse mammary glands this task no longer seems particle.

Technical Objective 3. Alter expression of receptors and growth factors to determine role in regulation of ErbB2 signaling and breast cancer.

Task 8. Place long-term implants (3-6 months) into ErbB2 overexposing mice. Pellets will contain possible ErbB2 regulating growth factors identified in Task 7. Analyze effects on ErbB2 signaling and tumor development.
Status: Due to expense of growth factors and mosaic expression of the ErbB2 transgene in mouse mammary glands this task no longer seems particle.

Task 9. Construct and subclone dominant negative receptor mutants with MCMV IE promoter into retroviral vector. Receptor selection based upon results from Aim 1.
Status: An alternative stately has been developed. See status of Task 11.

Task 10. Analysis of dominant negative mutants for activity in cell lines which express receptors.
Status: An alternative strategy has been developed. See status of Task 11.

Task 11. Express dominant negative mutants in mammary epithelium of ErbB2 overexposing mice and analyze effects on ErbB2 signaling and tumor development.
Status: An alternative stately has been used to identify the roles of ErbB2 and its signaling partners in normal and malignant mammary development. Transgenic mice expressing dominant negative ErbB2 and ErbB4 have been described in this report. A similar strategy has been used to analyze EGFR signaling (42) and I will examine ErbB3 signaling using transgenic expression of dominant negative receptors.